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A COMPARATIVE STUDY OF GASTRIC INTRINSIC FACTOR BINDING OF VITAMIN B_{12}

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A comparative study using affinity purified gastric intrinsic factor (IF) was carried out to determine the physicochemical differences between human, rat and rabbit IF. Purified IF from each source was bound to vitamin B_{12} and taken for proteolysis, paper chromatography, gel filtration, electrophoresis and immunoprecipitation studies under identical experimental conditions. Purified IF complexed with [^{57}Co]-vitamin B_{12} was also used in the experiments. Results of the study indicated inherent differences in the molecular nature of IF from these three sources. The observed differences were (1) in the manner of vitamin B_{12} binding and release by IF from each source, (2) in the absence of common epitopes on IF from rat and rabbit capable of reacting with polyclonal rabbit antihuman IF-B_{12} antisera, (3) in the amount of vitamin B_{12} released when IF-B_{12} was proteolysed by gastric and pancreatic proteases., and (4) by the lack of immunological cross reactivity and sequence homology between human, rat, rabbit, and hog IF. Release of B_{12} from proteolysed IF-B_{12} suggested that vitamin B_{12} release was not dependent on the action of a specific ileal releasing factor, as had been reported earlier.

Key words: IF-B_{12} binding, Proteolysis, Species specificity, Immuno complex.

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INTRODUCTION

Gastric intrinsic factor (IF) confers a unique status to B_{12} as being the only vitamin known to have a carrier mediated transport mechanism in the gastrointestinal tract of man and animals. IF exhibits species specificity, as a result, the clinical use of IF in subjects diagnosed for vitamin B_{12} deficiency states remains restricted to the use of hog IF. (1-3). Species specificity results due to molecular differences in the synthesis and secretion of genetically variant forms of this carrier protein by the gastric cells of different species. Endogenous differences in the nature of IF molecule produced by each of the three different species, were therefore investigated under identical experimental conditions. This comparative study is restricted to (a) purifying IF from human, rat and rabbit gastric sources using vitamin B_{12}-sepharose affinity matrix, and (b) assessing the molecular nature of purified IF from each source employing chromatography, electrophoresis and immune reactivity of both native and proteolysed IF-B_{12} complexes.

MATERIALS

Radioactive [^{57}Co] cyanocobalamin, (vitamin B_{12}, 1mci/mg, and 281 mci/mg) and radioactive Na^{125}I (4mci) were obtained from the Bhabha Atomic Research Center, Mumbai, India.

Sepharose-4B, and Blue dextran were obtained from Pharmacia, Uppsala, Sweden. Cyanogen bromide, pepsin, trypsin, a-chymotrypsin, carboxypeptidase -A and leucine aminopeptidase were obtained from Sigma Chemical Co, Mo, USA. All other chemicals used were of analytical grade from Indian manufacturers.

METHODS

Collection of human gastric juice

Histamine stimulated human gastric juice was collected by nasogastric suction from subjects who underwent this procedure as part of their clinical workup. The collection of gastric juice was made on ice and depepsinized immediately by raising the pH to 10.0 with 5N NaOH, allowing it to stand for 30 min. and then readjusting the pH to 7.5 with 1N HCl. The depepsinized gastric juice was centrifuged at 10,000 x g for 30 min. in a Sorvall RC - 2B refrigerated centrifuge. The supernatant obtained was kept frozen in aliquots for further use.

Preparation of rat and rabbit gastric mucosal extract

Rat and rabbit gastric mucosal extracts were prepared using a procedure essentially as described by Cooper and Castle (4). The animals were given drinking water ad libitum, and their stomach was removed under ether anesthesia. The organ was cut open and washed immediately (using cold 1.15% KCl for the rat stomach, and 0.02M sodium phosphate buffer for rabbit stomach) and the glandular region of the stomach was scraped using a glass slide. The scrapings were taken in 0.1M sodium phosphate buffer, pH 7.5 and homogenised in a Waring blender for 3-5 minutes to obtain a 20% (w/v) homogenate. The homogenate was centrifuged for 30 min at 25,000 x g in an MSE Mistral 6L refrigerated centrifuge at 4°C. The supernatant obtained was decanted, depepsinised and stored frozen for use as the rat IF source.

Assay for vitamin B_{12} binding ability:

The vitamin B_{12} binding ability of human, rat and rabbit IF was determined using the rapid in vitro method described by Gottlieb et al (5). Suitable controls containing protein coated charcoal and free radioactive [^{57}Co]-vitamin B_{12} were set up in parallel. Following centrifugation for 10 min. at 3500 x rpm in a tabletop clinical centrifuge, the control supernatant generally retained less than 1% of the initial total radioactivity of free vitamin B_{12}. The zirconium Phosphate gel (z-gel) at pH 5.0 (6) for estimating the vitamin B_{12} binding ability of a gastric IF preparation was also carried out. This assay can be used to adsorb IF–B_{12} complex from a sample quantitatively, to z-gel at pH 6.25 (7) employing type II serum autoantibodies to IF. This method was therefore adopted for the studies reported here.

Protein estimation

Protein in all the samples was estimated by the method of Lowry et al (8).

Purification of intrinsic factor from human, rat and rabbit sources

Intrinsic factor from depepsinised human gastric juice, rat and rabbit gastric mucosal extracts, was purified from the samples prepared and stored frozen earlier, using vitamin B_{12}-sepharose (1 ml gel) affinity chromatography (9). A sample of the IF source was thawed and applied to the gel followed by column washing and elution of the IF protein using established (9) procedures. Purified IF from each source was saturated with vitamin B_{12} for use in studies reported here.

Polyacrylamide disc gel electrophoresis

Electrophoresis was carried out using the purified protein on polyacrylamide gels (7%) by the method of Baenziger (10). The

electrophoretic run was conducted in the cold at 4°C using 0.10M sodium phosphate buffer, pH 7.4. Samples taken for electrophoresis contained protein (0.04mg/ml rat IF-B_{12}, 0.66mg/ml rabbit IF-B_{12} and 0.11mg/ml human IF-B_{12}) in 3% SDS and 1%, 2-mercaptoethanol to which bromophenol blue (0.5%) was added as a tracking dye. Following electrophoresis, gels were stained using 0.25 % coomassie blue in 10% methanol for 18 hr. Destaining was accomplished using 7 % acetic acid in 10% methanol for additional 18 hr.

Proteolysis of the IF-B_{12} complex

The effect of gastric and pancreatic proteases on purified human, rat and rabbit IF-B_{12} complexes was assessed by treatment of each sample in 0.1M sodium phosphate adjusted to contain pepsin at pH 1-2, followed 2 hr later with the addition of trypsin, a-chymotrypsin, carboxypeptidase-A and leucine aminopeptidase, at pH 7-8, allowing the reaction to proceed for a further 4 hr. The enzyme to protein ratio was maintained at 1:100 during the reaction at 37°C.

Gel filtration on Biogel P-60

A column (60 cm x 0.6 cm) of Biogel P-60 was prepared at room temperature and equilibrated with 0.1M potassium phosphate buffer, pH 7.4., having a flow rate of 7.5ml/hr. Protein samples containing [^{57}Co] vitamin B_{12} were applied to the column, at a constant flow rate was maintained using a peristaltic pump. Fractions of I.0 ml were collected employing an LKB Redirac fraction collector and the radioactivity in each fraction was measured using a Packard gamma counter. Void volume (V_0) of the column was determined using 0.5 ml of 1% solution of blue dextran 2000.

Paper chromatography of the IF-B_{12} Complex

The IF-B_{12} complexes, native and proteolysed, containing bound [^{57}Co]-B_{12} were chromatographed on a whatman no. 1 filter sheet (size 56.5 cm x 22.5 cm) employing isopropanal/water solvent system (4:1 ratio) at room temperature. The chromatogram was developed for 18hrs by the descending method and dried in air. The dried chromatogram was sprayed with a solution of ninhydrin (0.4%) in acetone and dried (60°C, 15min) to observe the purple products of proteolysis, as well as the native complexes.

Immuno diffusion studies using rabbit antiserum to purified human IF-B_{12} complex

Polyclonal rabbit antibodies to purified human IF-B_{12} complex (containing 350 ug IF protein saturated with 8.05 ug B_{12}) was generated in an adult, normal, healthy, male rabbit using a sample (1.0 ml) of purified human IF-B_{12} complex, mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into the dorsal skin of a rabbit in several spots. The rabbit was maintained on normal diet for 15 days. A booster dose of the IF-B_{12} complex (100 ug protein in 0.3 ml mixed with Freund's adjuvant) was then administered to the rabbit on day 16. Blood was collected from the ear lobe of the animal 3rd day following the booster dose, serum separated and stored frozen as the antibody source. Immunodiffusion experiments were carried out using the polyclonal rabbit anti humanIF-B_{12} antiserum against purified human, rat and rabbit IF-B_{12} forms and hog IF (Sigma), employing the method of Ouchterlony (11).

RESULTS

The amount of total protein in each source applied to the affinity gel matrix, and the amount of purified IF recovered following each column operation is as given (Table 1). The vitamin B_{12} binding capacity and the specific activity of each source is also given. Differences in the quantitative yield of the IF protein from each source following column operation and differences in their specific activities were observed. The purified IF was however found to be electrophoretically homogenous (Fig.1).

Human IF was purified 1035 fold, rat IF 1729 fold, and the rabbit IF 116 fold. Purified human IF exhibited three fold greater specific activity than that of the purified rat IF, and purified rabbit IF showed two fold lesser specific activity than that of the purified rat IF. The yield of total vitamin B_{12} binding capacity of human IF and rabbit IF were closely similar, whereas that of rat IF was 17% lower.

A plot of radioactivity from a sample of [^{57}Co]-vitamin B_{12}, native and proteolysed, subjected to paper chromatography and detected based on the radioactivity distribution in one cm width pieces of the paper strips, showed differences in the mobilities of the native and the proteolysed IF-B_{12} complexes (Table 2).

The Biogel P-60 column elution profile of each IF-B_{12} complex is as shown in the figure (Fig 2-4). Native IF-B_{12} of human, rat and rabbit yielded two peaks of [^{57}Co]-B_{12} radioactivity The position of peak 1 related to protein bound radioactivity and that of peak 2 related to the unbound [^{57}Co]-vitamin B_{12}. Proteolysed human and rabbit IF-B_{12} also yielded two radioactive peaks as for the native complex. Proteolysed rat IF-B_{12} however, yielded only a single peak of radioactivity. A quantitative decrease in the protein bound [^{57}Co]-B_{12} radioactivity was also observed (Table 3) whenever the proteolysed IF-B_{12} forms were employed.

Vitamin B_{12} binding by human gastric juice, rat and rabbit gastric mucosal extracts exhibited first order kinetics. The vitamin B_{12} binding capacity of each was saturable. Quantitative differences in the capacity of each IF to bind vitamin B_{12} (tables 1-3) was noted. Differences were observed in their reaction times also. Human gastric juice required 15 min. for saturation of its B_{12} binding ability (12) whereas rat IF required 5 minutes, and rabbit IF required only 10 seconds (12).

Cross reactivity between the polyclonal rabbit antisera and the IF-B_{12} complexes of rat and rabbit and the sample of pure hog IF (Sigma) was not observed during the Ouchterlony immuno diffusion experiments (Table 4). Results of the zirconyl phosphate gel assay however, showed that only the human IF-B_{12} was capable of being adsorbed onto the gel at pH 6.25 (Table 5).

DISCUSSION

The gastric glycoprotein IF having a molecular weight of 60 kDa and reportedly (13) containing a single site for vitamin B_{12} binding, when purified from human, rat and rabbit gastric sources employing vitamin B_{12}-sepharose affinity chromatography yielded electrophoretically homogenous IF from each source but having significant differences in their final specific activities. The chromatographic studies established that proteolysed human and rabbit IF retained bound B_{12} during the gel filtration process. However, fragility of B_{12} binding by rat IF was observed following proteolysis and gel filtration of the proteolysed rat IF-B_{12}. Nonetheless, retention of bound B_{12} by human IF following proteolysis was also noted due to the presence of [^{57}Co]-B_{12} radioactivity in the ninhydrin spots that coincided with the protein mobility. Furthermore, electrophoretic mobility differences existed between the native human IF-B_{12} and the proteolysed human IF-B_{12} complex. Molecular weight estimates yielded an $\underline{M}^r$=60 kDa for both types of complexes, however.

Z-gel assay detected the loss in type II antibody (7) binding epitope of human IF following the protease action. The presence of the type II epitope in the IF polypeptide is suggested to correlate with the ability of IF-B_{12} to recognize and bind to its' specific ileal receptors (9). The invitro generation of proteolysed IF-B_{12} complex, lacking the type II epitope exemplifies the presence of the modified IF-B_{12} form in the gastrointestinal tract of man and animals.

Cross-reactivity studies did not suggest immunocomplexation of rat, rabbit and hog

Table 1 Purification of Intrinsic Factor

Affinity step	Sample	Total volume ml	Total activity µg B_{12}	Total Protein mg	Specific activity ug B_{12}/mg protein	Yield %	Fold purified
A	Celite filtrate of HGJ	1080	42.12	1890	0.022	100	1
	Guanidine HCl eluate (HIF)	10	23.19	1.05	22.77	57	1035
B	Rat gastric mucosal extract	66.0	0.944	11.55	0.008	100	1
	Guanidine HCl eluate (RatIF)	0.85	0.448	0.0324	13.83	48	1729
C	Rabbit gastric mucosal extract	96.0	2.9	816	0.0036	100	1
	Guanidine HCl eluate (RbIF)	6.2	1.91	4.02	0.466	65	116

Table 2 Paper Chromatography of IF - B_{12} complex

Sl. No	Sample type	Specific activity µgB12/mgIF)	Specific radioactivity µci / μgB_{12}	Volume applied µl	[^{57}Co]-B_{12} (CPM)	R_f
A	Free vitamin B_{12} spiked	-	-	20	1000	0.58
B	**Native complexes**					
1	Human IF – B_{12}	24	3.5×10^{-4}	50	100,000	0.58
2	Rat IF B_{12}	13.8	9×10^{-4}	50	20,500	0.59
3	Rabbit IF – B_{12}	0.5	2×10^{-3}	100	291,000	0.58
C	**Proteolysed Complexes**					
1	Human IF - B_{12}	24	3.5×10^{-4}	50	22,300	0.79
2	Rat IF - B_{12}	13.8	9×10^{-4}	50	41,000	0.66
3	Rabbit IF - B_{12}	0.5	2×10^{-3}	100	39,000	0.67

Table 3 Gel filtration using Biogel P-60

Sample type	Volume applied	Total ^{57}[Co]-B_{12} radioactivity applied to column	Number of radioactive peaks eluted
Native complex	(ml)	(cpm)	
Human IF - B_{12}	0.4	131,000	2
Rat IF - B_{12}	0.15	42,000	2
Rabbit IF - B_{12}	0.20	85,400	2
Proteolysed complex			
	0.40	40,000	2
Human IF - B_{12}	0.15	42,000	1
Rat IF - B_{12}	0.30	120,000	2
Rabbit IF - B_{12}			

Table 4 Ouchterlony immunodiffusion studies

Sample	Volume (ml)	Sample protein (ug)	Antisera Protein (mg)	Result
Human gastric juice (well 1)	50	3.0	3.4	Positive
Unproteolysed human IF-B_{12} (well 2)	10-50	2.65 – 13.25	4.4	Positive
Proteolysed human IF-B_{12} (well 3)	10-50	20 – 10.0	4.4	Negative

Table 5 Zirconium phosphate gel assay

Sl. No.	Sample	Z-Gel at pH 5.0 without antiserum	Z-gel at pH 6.25 with rabbit antihuman IF-B_{12} antiserum
1	Human IF-B_{12}	+	+
2	Rat IF-B_{12}	+	-
3	Rabbit IF-B_{12}	+	-

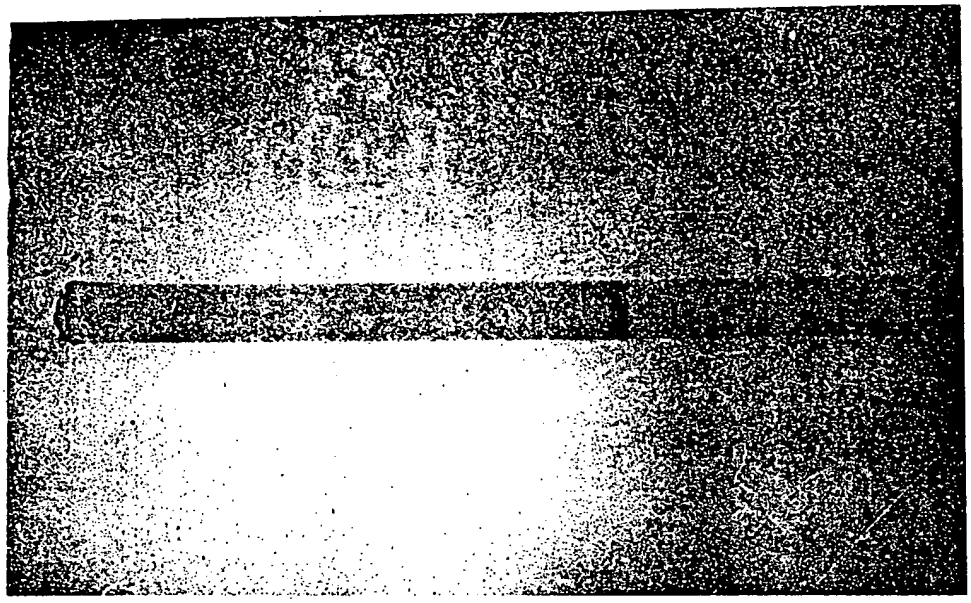

Fig.1 SDS-polyacrylamide disc gel (7%) electrophoresis of affinity purified rabbit IF-B_{12} carried out for 90 min using 4 milliamps current per tube gel, as described under methods. The coommassie blue stained homogenous protein band is seen (arrow). Affinity purified human and rat IF-B_{12} yielded homogenous protein bands similarly.

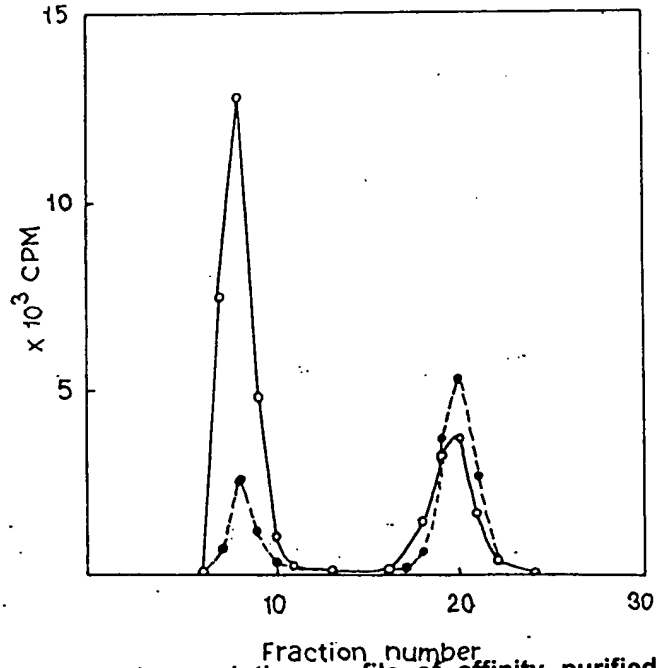

Fig. 2 Biogel-P60 column elution profile of affinity purified human IF-[^{57}Co]-B_{12} based on the B_{12} radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B_{12} radio activity, and Peak 2 represents free B_{12} radioactivity. Solid line: human IF-B_{12}, Dashed line: proteolysed human IF-B_{12}

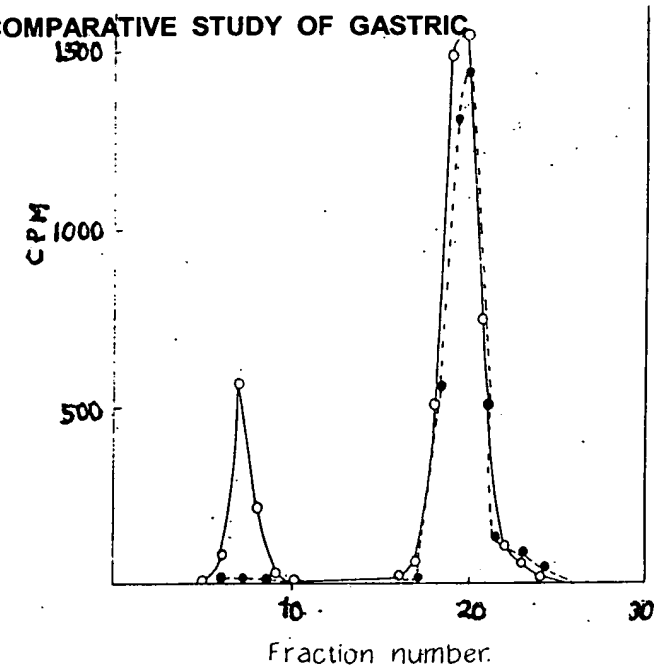

Fig. 3 Biogel-P60 column elution profile of affinity purified ratIF-[^{57}Co]-B_{12} based on the B_{12} radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B_{12} radio activity, and Peak 2 represents free B_{12} radioactivity. Solid line: ratIF-B_{12}, Dashed line: proteolysed rat IF-B_{12}

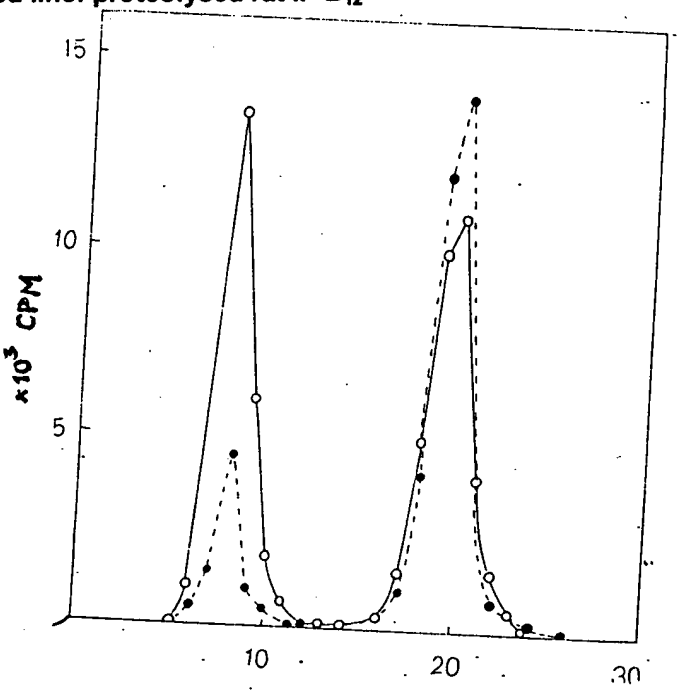

Fig. 4 Biogel-P60 column elution profile of affinity purified rabbit IF-[^{57}Co]-B_{12} based on the B_{12} radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B_{12} radio activity, and Peak 2 represents free B_{12} radioactivity. Solid line: rabbit IF-B_{12}, Dashed line: proteolysed rabbit IF-B_{12}

IF with the polyclonal rabbit antihuman IF-B_{12} antiserum, pointing to the lack of immunological sequence similarities between human, rat, rabbit or the hog IF. Lack of immunoreactivity by proteolysed human IF-B_{12} with the rabbit antiserum suggested structural changes in the IF-B_{12} that resulted in the loss of its'antibody binding ability.

The observed differences in the IF binding of vitamin B_{12} may result from, (1) the interaction of other proteins in the gastric IF source with the affinity matrix, reducing the IF binding capacity of the matrix competitively, and thereby affecting the final yield of the purified IF from each source, (2) the nature of non-specific interactions by the glycosyl moiety of IF with other proteins in a sample, may yield conformationally restricted IF polypeptides, remaining incapable of interacting with the B_{12} ligands of the affinity matrix, and (3) the use of 7.5M guanidine hydrochloride that led to partial denaturation of the IF from different sources and resulted in the reduced vitamin B_{12} binding ability of the purified IF.

Using partially purified extracts from rat intestines complexed with B_{12}, Rahman (14) observed the kinetics of B_{12} release to be of an enzymatic type. Ungley (15) proposed and others (16-19) suggested the existence of a 'releasing factor', in the small intestines of man and animals capable of removing vitamin B_{12} from the IF-B_{12} complex. Cooper and Castle (4) had confirmed such a proposal using rat intestinal extract and rat IF concentrate. Our studies utilized proteolysis of purified human, rat and rabbit IF-B_{12} complexes to suggest (a) that structural alterations to IF occurred as a consequence of proteolysis, and (b) that such alterations released bound vitamin B_{12} (50-70%) from IF.

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